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## Note

# Simple and direct determination of pyridinoline and 2'-deoxypyridinoline by automatic analysis

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Since discovery of pyridinoline[1] and 2'-deoxypyridinoline[2] crosslinks in collagen molecules, experimental evidence[3,4] suggests that they are age-dependent products in metabolic pathways which involve reducible crosslinks, and that the content varies broadly with the type of tissue. The sparse occurrence of the crosslinks in these tissues, however, makes it very difficult to determine their content in collagen unless time-consuming enrichment is carried out by Biogel P-2 or phosphocellulose column chromatography. This paper describes an elaboration of the elution system for a simple and effective method of determination of pyridinium crosslinks in collagen hydrolysate.

## MATERIALS AND METHODS

A standard amino acid mixture and reagents were obtained from Wako. Bovine Achilles tendon was a product of Worthington Diagnostic System. Pyridinoline and 2'-deoxypyridinoline were prepared from bovine bone insoluble collagen[5] by the previous method[2]. Desmosine and isodesmosine were isolated from elastin of bovine *ligamentum nuchae* according to the method of Partridge *et al.*[6], after pre-concentration of desmosines on a cellulose column[7].

An analytical standard solution was made up to contain *ca.* 10–20 nmol of pyridinium crosslinks and 60 nmol of each amino acid in 1 ml of 0.01 *M* hydrochloric acid.

Insoluble collagen was hydrolysed in 6 *M* hydrochloric acid in a sealed tube under nitrogen for 24 h at 110°C. The hydrolysate was rotary-evaporated to dryness and redissolved in 0.01 *M* hydrochloric acid. Amino acid analysis was then carried out with a Hitachi Model 835 analyser fitted with a sampling valve with a 50-$\mu$l loop. Single-column analyses were normally performed at 53°C on a 150 × 4 mm I.D. column of sulphonated polystyrene resin (10% divinylbenzene, 7 $\mu$m), Hitachi No. 2619. The flow-rates were set to 0.225 ml/min for buffer and 0.3 ml/min for ninhydrin. The buffers and the ninhydrin reagent were prepared according to the Hitachi technical manual, except Buffer 5. All the sodium citrate buffers contained 0.4% (v/v) Brij-35 and 0.01% (v/v) octanoic acid. Buffers 1, 2, 3 and 5 contained 0.5% (v/v) thiodiglycol. Buffers 1 and 2 contained 13% and 2% (v/v) ethyl alcohol, respectively.

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TABLE I

ELUTION SYSTEM FOR THE SEPARATION OF PYRIDINIUM CROSSLINKS FROM AMINO ACID MIXTURE

Programme: 0–1 min, Buffer 1; 1–8 min, Buffer 2; 8–25 min, Buffer 3; 25–33 min, Buffer 5; 33–50 min, Buffer 4; 50–56 min, Buffer 6; 56–60 min, Buffer 2; 60–82 min, Buffer 1.

| *Buffer no.* | *Composition* |
|---|---|
| 1 | 0.2 *M* ($Na^+$) Citrate (pH 3.3), 13% ethyl alcohol |
| 2 | 0.2 *M* ($Na^+$) Citrate (pH 3.3), 2% ethyl alcohol |
| 3 | 0.2 *M* ($Na^+$) Citrate (pH 4.3) |
| 4 | 1.2 *M* ($Na^+$) Citrate (pH 4.9), 0.5% benzyl alcohol |
| 5 | 0.2 *M* ($Na^+$) Citrate (pH 5.28) |
| 6 | 0.2 *M* Sodium hydroxide |

Buffer 5 contained 0.5% (v/v) benzyl alcohol. Table I summarizes the operating conditions. To obtain optimum resolution of pyridinoline and 2′-deoxypyridinoline from the other amino acids in the hydrolysate, an elution system was adopted with the addition of a buffer (5) between Buffers 3 and 4.

## RESULTS AND DISCUSSIONS

As shown in Fig. 1, the pyridinium crosslinks are clearly separated in distinct peaks with very fine resolution from those due to the other amino acids, and from desmosine and isodesmosine if there is contamination by elastin. In fact, even an excess sample charge (0.75 mg of hydrolysate in a loop, *ca.* 150 times as large as usual) allows a satisfactory determination of pyridinoline and 2′-deoxypyridinoline (Fig. 2). Thus, we can determine the amino acid and pyridinium crosslink composition with a single elution system by sequentially putting the usual and concentrated samples into an autosampler system if necessary. A linear response of pyridinolines with the ninhydrin reaction was obtained in the range 0.5–10 nmol (Leu equivalent). The detection limit is usually below 0.3 nmol per injection, and depends on the purity

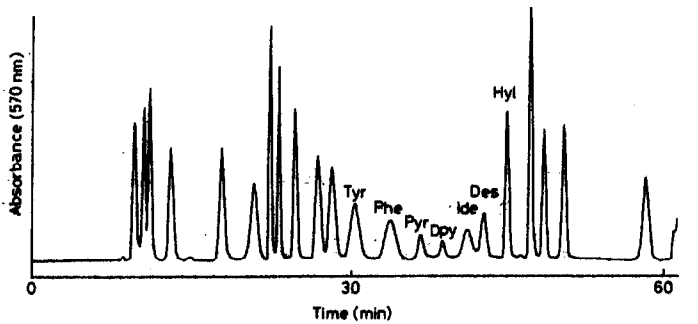


Fig. 1. An amino acid chromatogram for the analytical standard. Each amino acid (3.0 nmol per injection): Pyr = pyridinoline (0.62 nmol Leu equivalent); Dpy = 2′-deoxypyridinoline (0.39 nmol Leu equivalent); Ide = isodesmosine (0.91 nmol Leu equivalent); Des = desmosine (1.1 nmol Leu equivalent).

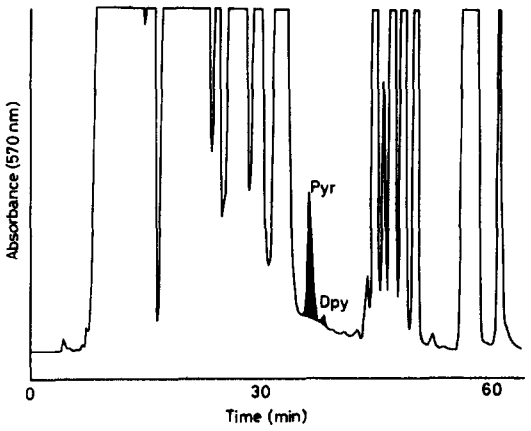


Fig. 2. An amino acid chromatogram for the concentrated hydrolysate of bovine Achilles tendon (*ca.* 0.75 mg per injection).

of collagen samples and the content of crosslinks in collagen. The method needs only 80 min for a run and precisely determines the pyridinium crosslinks in the collagen hydrolysate using a conventional analyser system with ninhydrin detection. It can be conveniently applied in connective tissue research, for example on age or pathological changes in bone, tendon, ligament, and aorta.

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